Patent
Attorney's Docket No. 1017753-000235

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	**Mail Stop AMENDMENT**
Bertrand PAIN et al.	Group Art Unit: 1633
Application No.: 10/625,847	Examiner: S. Kaushal
Filed: July 24, 2003	Confirmation No.: 8939
For: AVIAN CELL LINES USEFUL FOR THE PRODUCTION OF SUBSTANCES OF INTEREST	

DECLARATION UNDER 35 U.S.C. § 1.132 of DR. MAJID MEHTALI

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Majid Mehtali, declare the following:

2. I am a citizen of France, and have the following mailing address: Le Mortier 44, Couéron, France;

3. I graduated from University Louis Pasteur with a Ph.D. degree in Biotechnological Engineering;

4. I am the Chief Scientific Officer of Vivalis, and have held this position since the year 2003;

5. I am skilled in the art of developing cell lines for the production of biologicals, such as viral vectors, vaccines and recombinant proteins;

6. I have read and am familiar with the above-identified United States Patent Application, and I am submitting this Declaration in support of that application;

7. Chicken cell lines have been obtained by a process equivalent to the process described for the Duck 26 Ebx cell line in Example 2 of the present Declaration, and the process results in Chicken EBx cell lines having the same essential characteristics as those shown in the present Declaration with regard to the Duck 26 Ebx cell line;

8. I have performed and/or supervised the experiments reported below:

1. INTRODUCTION

A large number of human and veterinarian recombinant and non-recombinant vaccines are currently produced on embryonated eggs or alternatively on primary fibroblasts isolated from chicken embryos (Table 1). Although being reliable, the current egg-based production system is associated with serious limitations, including: (1) a lengthy and cumbersome manufacturing process; (2) the need in many cases to use specific pathogen free (SPF) chicken embryos; (3) the requirement of hundreds of millions of fertilized chicken eggs to manufacture the vaccines, in particular in the case of the human influenza vaccine; (4) the allergenicity of eggs in some individuals and (6) the inability to use eggs for the propagation of viruses that are highly virulent and lethal to chickens.

Table 1: animal and human vaccines produced on chicken egg-based systems

AVIAN	SWINE	EQUINE	HUMAN	RECOMBINANT
influenza virus	influenza virus	influenza virus	influenza virus	Canarypox
reovirus		Eastern equine encephalomyelitis	Yellow fever virus	Fowlpox
fowlpox virus		Western equine encephalomyelitis	measle virus	Modified Vaccinia Virus Ankara (MVA)
canarypox virus			Mumps virus	Alphavirus - Sinbis virus
chicken poxvirus			Rabies	Alphavirus - Semliki Forest Virus
psittacine herpes virus			Smallpox	Alphavirus - Venezuelan EEV
Newcastle Disease Virus				Avian Adenovirus - CELO
falcon herpes virus				
pigeon herpes virus				
infectious bursal disease virus				
infectious bronchitis virus				
Marek's disease virus				
turkey herpes virus				
chicken anemia virus				
avian encephalomyelotis virus				
polyomavirus type I & II				
Adenovirus type I, II & III				

Therefore the development of a safer and faster cell-based production system is urgently needed, as regularly stated by numerous national (*e.g.* FDA) and international (*e.g.* WHO) organizations. The development of a stable and well characterized cell line for the production of viral vaccines is highly desirable tool for human and animal health vaccine manufacturers. Ideally, such a cell line should have the following characteristics :

- full characterization
- known history

- no step of immortalization by genotoxics or genetic modifications
- non-tumorigenic
- stable under long-term culture conditions
- ability to grow in suspension
- efficient growth in absence of serum
- High viral yields
- Reproducible process

Based on these requirements, and given the fact that many different viruses were historically replicated in chicken embryo fibroblasts, the Applicant, VIVALIS, has taken advantage of its unique expertise in avian biology and more specifically in avian embryonic stem cells, to undertake the development of novel proprietary avian cell lines (i.e the EBx® platform) that fulfill the needs of the vaccine industry. In addition, such cellular substrate is ideal for the production of a recombinant protein of interest, such as a monoclonal antibody.

2. VIVALIS EBx® CELLS: AN ALTERNATIVE CELL-BASED VACCINE PRODUCTION PLATFORM

VIVALIS established a two-step proprietary process to derive stable adherent and suspension cell lines, named EBx®, from avian embryonic stem cells (ES) cells with no steps of genetic, chemical or viral immortalization (Fig.1). This process includes:

- 1) the *in vitro* culture and expansion of avian ES cells (Step 1)
- 2) the derivation of EBx® cells from avian ES cell culture (Step 2):

2.1 - Step 1: In vitro culture and expansion of avian ES cells

Embryonic stem (ES) cells are unique in that: (i) they can self-renew indefinitely *in vitro* as undifferentiated cells, (ii) they have unlimited regenerative capacity, (iii) they maintain a stable chromosomal content; (iv) they express high levels of telomerase and specific cell-surface markers. Despite many efforts worldwide, ES cells have been successfully isolated from only a very limited number of species (mouse, human, monkeys).

The inventors have dedicated significant resources over the last years to isolate and establish ES cells from different avian species, and mainly from different chicken strains and duck. Such research efforts led to the successful isolation and characterization of chicken ES cells and duck ES cells.

The inventors were able to isolate and culture chicken ES cells of all the chicken strains tested (Table 2), while maintaining their undifferentiated phenotype. Valo and S86N strains were further used to establish EBx® cell lines because of the good sanitary status of such chicken strains.

Table 2: Isolation and culture of ES cells obtained from different strains of chicken:

Strain	Nb of experiments	Nb of isolates	% success
S86 N	127	33	26
Valo	17	5	29
Brown Leghorn	53	4	7
GF30	20	4	20
White Leghorm	14	1	7
Cou Nu Rouge	11	4	36
Marans	29	16	55
Barred Rock	**49**	**35**	**72**
ISA	3	1	33

The inventor then developed proprietary procedures that allow the efficient *in vitro* culture and large-scale expansion of chicken and duck ES cells without induction of differentiation.

The ultimate proof of the ES status of cultured avian cells is the demonstration of the totipotency of such cells. To do so, the inventors have realized an experiment to evaluate the ability of chicken ES cells maintained during more than 2 weeks into culture to contribute to in vivo reconstruction of a chicken embryo (See Appendix

N°1). Thus the inventors demonstrate the germ-line transmission of cultured chicken ES cells according to the process of the invention.

2.2 - Step 2: Derivation of EBx® cells

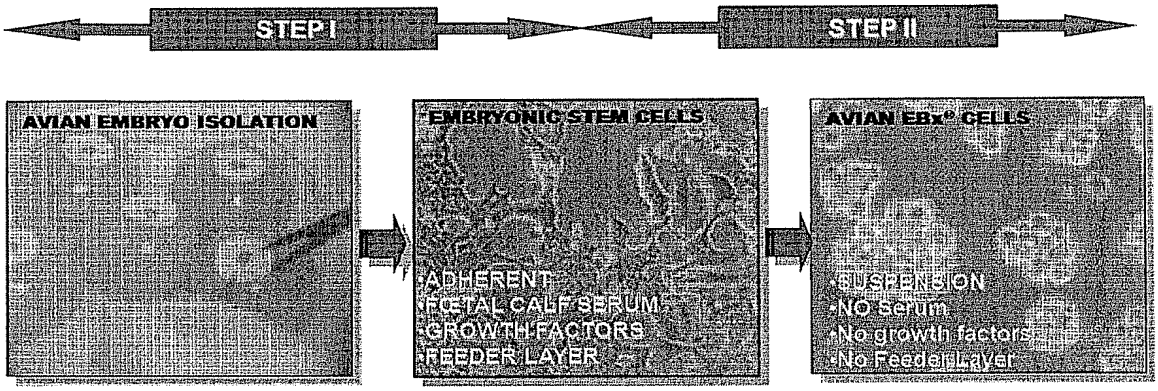
Then the inventor established a proprietary process to derive stable, adherent or suspension, continuous cell lines from avian ES cells. The process includes the long and progressive withdrawal of serum, feeder cells and growth factors from the cell culture medium and the adaptation of cells to a suspension culture. These embryonic derived avian cell lines, named EBx®, maintained most of the desirable features of ES cells (ie. indefinite proliferation, expression of ES specific markers such as the telomerase, stability of the karyotype) but in addition displayed new "industrial-friendly" characteristics (growth in suspension in serum-free media up to high cell densities).

This process of derivation of EBx® cells is reproducible and not limited to a specific chicken species. The inventors successfully generated chicken EBx® cells derived from ES cells obtained from different chicken strains (namely Valo and S86N45). The Appendix N°2 contains one example of a chicken EBx® cell line from Valo strain, named EBv13 established by the inventor. In addition, the inventors successfully generated duck EBx® cells derived from duck ES cells. The Appendix N°2 contains example of one duck EBx® cell line established by the inventor (see Example 2 of the Affidavit).

Based on their attractive biological properties, the inventors selected such EBx® cell lines for further development.

AC

Figure 1: Establishment of EBx cells from avian embryonic stem cells



Of particular importance, EBx® cells have conserved some essential "stem cell" features:

- They display an ultra-structure similar to embryonic stem (ES) cells (Fig. 2). This ultra-structure is conserved throughout cell passages as demonstrated by photonic and electronic microscopy analysis of EBx® cells at early and late passages. Analysis performed on the end of production cell bank at approximately passages 160 shows an ultra-structure of EBx® cells similar to embryonic stem; cells at passage 160 were approximately grown approximately 500 days into culture.
- They express a series of markers known to be specifically expressed in embryonic stem cells (*eg.* Alkaline phosphatase, SSEA-1, EMA-1, ECMA-7, telomerase), but not in differentiated cells such as the continuous avian DF1 fibroblast, hamster CHO or canine MDCK epithelial cell line (Fig. 3). The expression of the above cited markers known to be specifically expressed in ES cells was shown to be expressed in EBx® cells during more than 160 passages, which corresponds to approximately 500 days into culture. Expression of these markers is lost upon experimental induction of cell differentiation by addition of retinoic acid (RA) or DMSO.

- As a probable consequence of the strong expression of telomerase (Fig. 4 & 5), EBx® cells replicate indefinitely *in vitro* (Fig. 6); several candidate cell lines have been cultured for more than a year without differentiation. As a consequence, Master cell banks of chicken and duck EBx cells lines were produced in animal serum-free medium according good manufacturing practices (GMPs) at respectively passages p160 and p134.
- In addition, the analysis of telomerase over time showed a stable maintenance of the enzymatic activity (Fig.5), while no telomerase activity was detectable in differentiated avian DF1 fibroblasts, in CHO cells or in canine epithelial MDCK cells (Fig. 4).

Figure 2 : Chicken & duck EBx cells display an ultra-structure similar to embryonic stem cells.

EBx cells are small, round, individualized cells with a high nucleo-cytoplasmic ratio and display a similar ultra-structure to mouse embryonic stem cells. The cell morphology is kept throughout the passages.

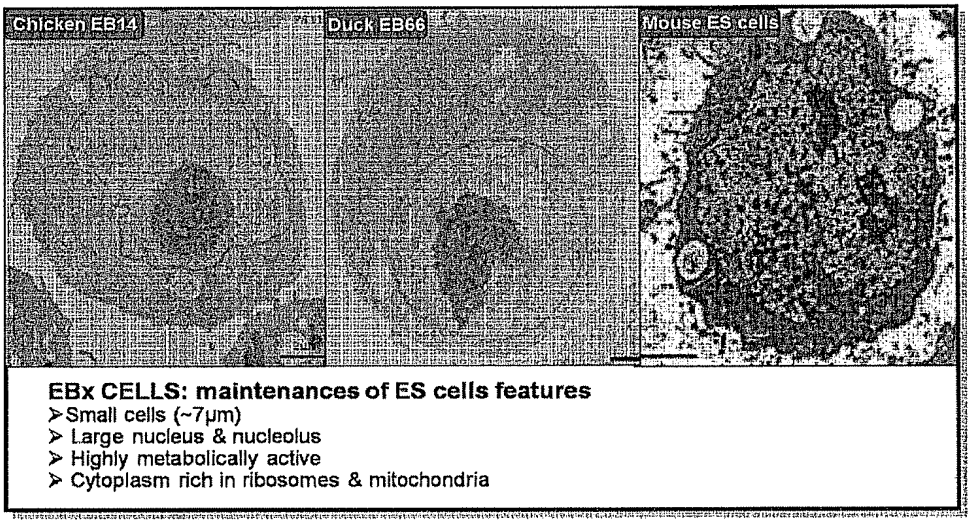


Figure 3 : ES cell-specific markers expressed on chicken EBx cells-

Cell surface markers were analysed by FACS analysis. Chicken EBx cells do express stem-cell specific markers like EMA-1, SSEA-1, ECMA-7. The chicken DF1

fibroblast cells used as a negative control do not express stem cell specific markers but ubiquitous marker like paxilline.

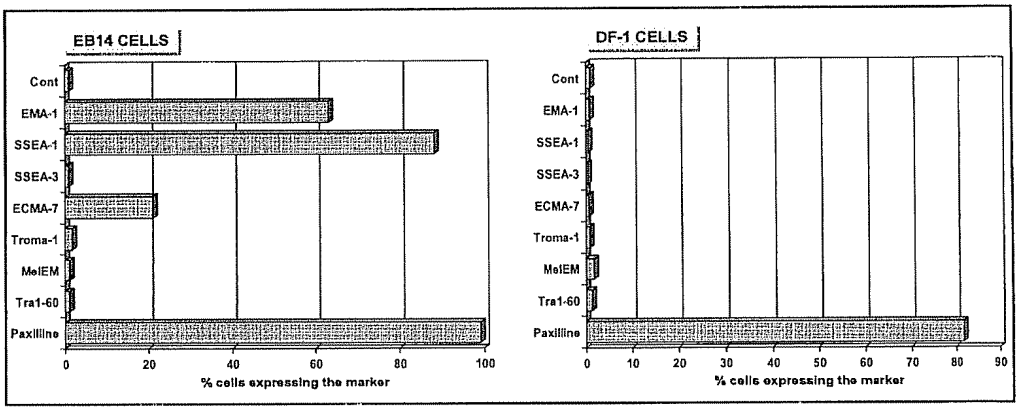


Figure 4: ES cell-specific markers:

Expression of telomerase in chicken and duck EBx cells and in differentiated chick fibroblasts, CHO and MDCK cells.

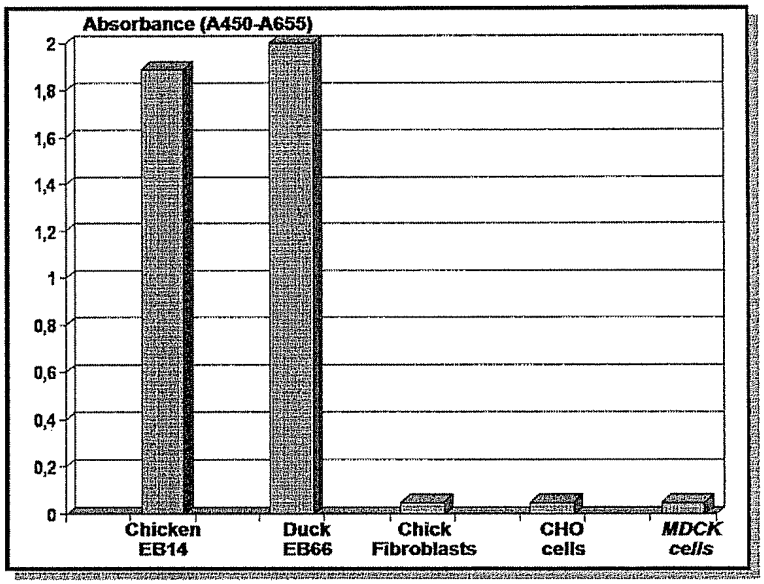


Figure 5: ES cell-specific markers:

Expression of telomerase in duck EBx cells is maintained throughout cell passages and sub-cloning. Duck EB24 and Duck EB24 subclones (EB24-12 & EB24-8) at different passages (P120, P146, P127) keep expressing high level of telomerase. Same observation can be done with other duck EBx cell lines (EB26 & EB66).

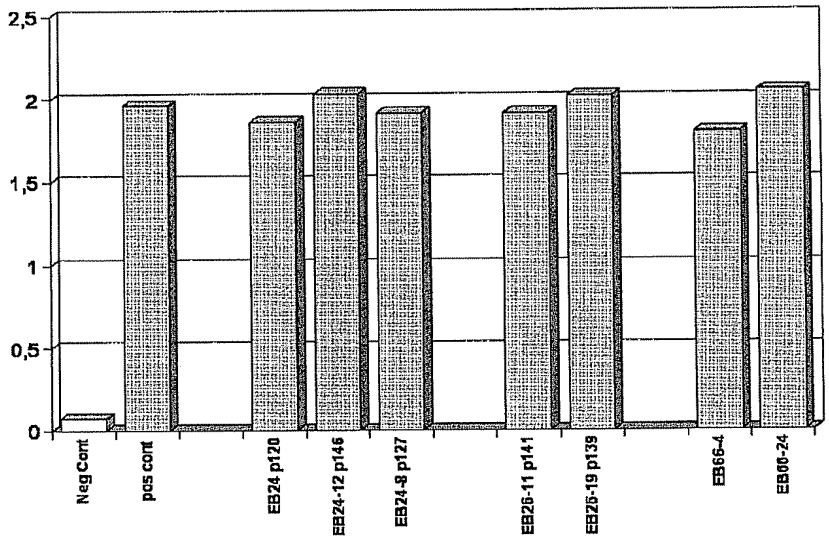
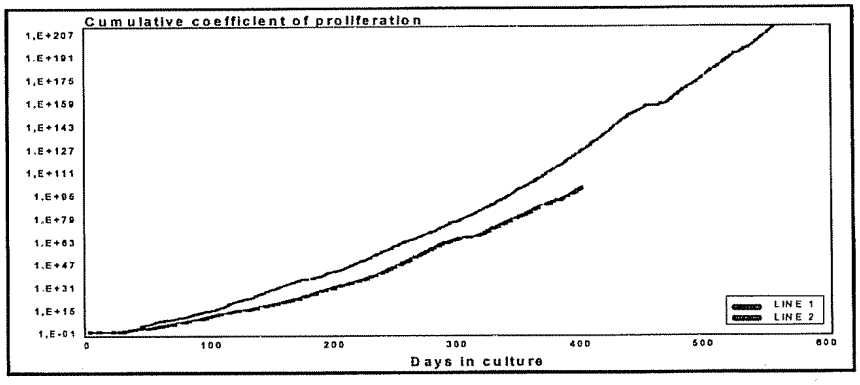


Figure 6: Long-term proliferation of EBx cells



Based on all these properties, the suspension of chicken and duck EBx cells have been selected by VIVALIS for further analysis of their growth characteristics and susceptibilities to various viruses.

3. EBx® CELL GROWTH KINETICS

Chicken and duck EBx cells have been analysed in detail for their cell culture characteristics. In particular, parameters such as the population doubling time, the temperature sensitivity, the influence of the medium, the adaptation to serum-free medium, the growth as suspension cells in spinner flasks and in bioreactors were more specifically investigated.

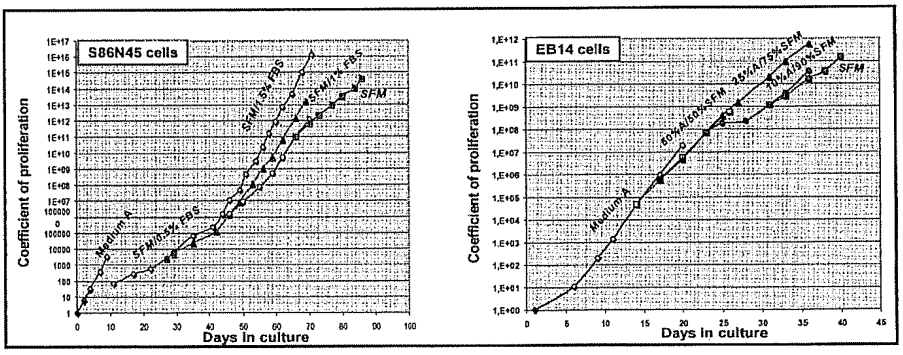
3.1. Population doubling time

Chickens and ducks have a relatively short population doubling time of approximately 17h at 37°C that are adequate for virus propagation (see below).

3.2 Adaptation to serum-free medium

Duck and chicken EBx cells were adapted to the growth in serum-free medium (SFM). Several serum-free medium formulations have been tested and a couple of serum-free medium formulations from commercial origin have been identified that allow the efficient growth of chicken and duck EBx cells (Fig.8).

Figure 8: Adaptation of chicken EBx cells to growth in serum-free medium

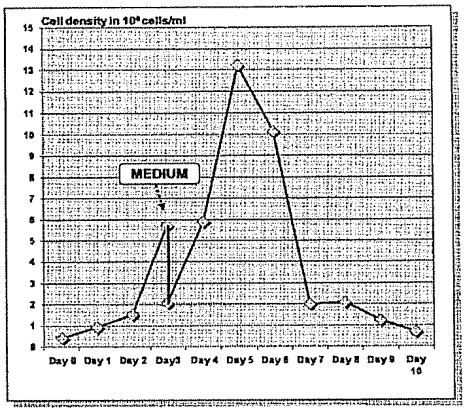


Growth in suspension in serum-free medium in Bioreactor

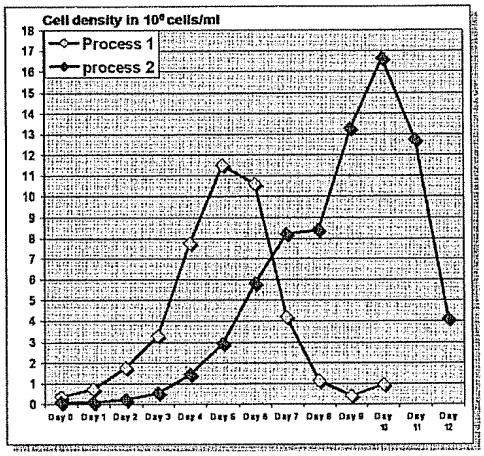
Duck and chicken EBx® cells are thus able to grow in suspension in serum-free and growth factors free medium in different cell culture vessels, such as tissue-culture flasks, spinner, Wave™ bioreactors and stirred-tank bioreactors (Fig.10).

Figure 10: EBx® cell growth in serum-free medium in 3L-bioreactor

10A) – Chicken EBx cells grow to high cell densities in serum-free medium (> 10 millions cells/ml). When fresh serum-free medium is added during the culture, EBx cells may reach higher cell densities.



10B)- Duck EBx® cells grow to high cell densities in serum-free medium (10-20 millions cells/ml). When nutrients are added during the culture, duck EBx cells may reach higher cell densities.



3.3 Stability of the EBx® cells karyotype

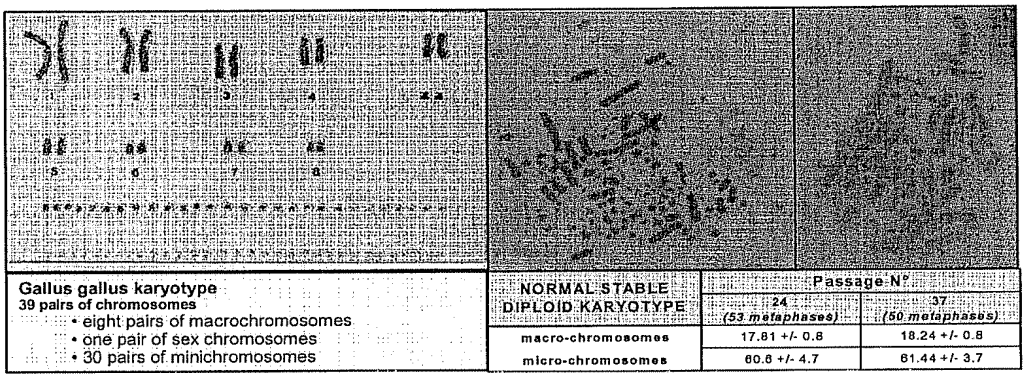
EBx® cells are immortal as a probable consequence of the stable and elevated expression of the telomerase (Fig. 4 & 5). It is therefore of particular importance to determine whether such cells are genetically stable upon long term cultures *in vitro*.

Suspension chicken and duck EBx cells have thus been grown in culture for several passages and 50 metaphases have been analyzed for their chromosomal content by Merial (Lyon, France) and Pr. M. Franck (ENVL, Lyon, France).

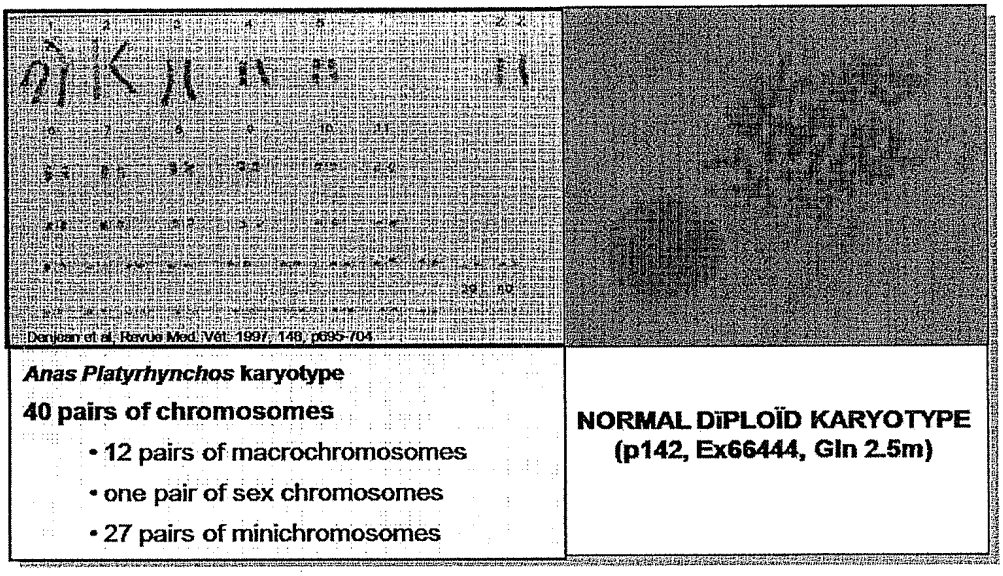
Analysis of chicken EBx cells confirmed the genetic stability of these cells since an average of 18 macrochromosomes (9 pairs) and 60 microchromosomes (30 pairs) were observed in this experiment at passages 24 and 37 (Fig. 11A). Analysis of duck EB66 cells karyotype also confirmed the diploidy and thus the genetic stability of these cells (Fig. 11B).

Figure 11: karyotyping of EBx® cells

11A) – Chicken EB14 cells



11B) – Duck EBx cells



Karyotype performed by Pr. Franck, ENVL, Lyon

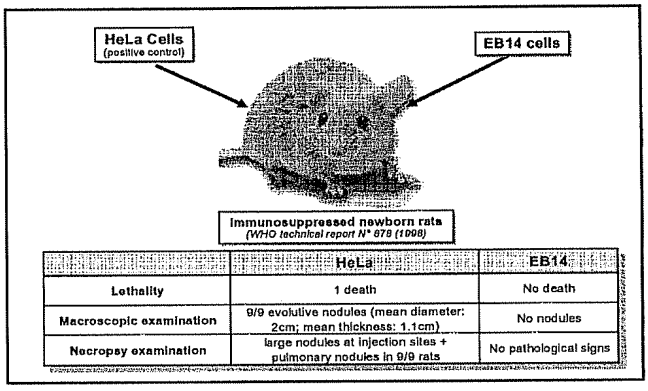
3.4 Tumorigenicity of EBx cells

The tumorogenicity of the cell substrate is a key issue, in particular for the production of live vaccines. The potential tumorigenicity of chicken EBx cells was investigated *in*

vivo by Merial and Aventis-Pasteur (Lyon, France) in an experiment in which 10 millions EBx cells were injected sub-cutaneously into 10 newborn immuno-suppressed rats. Human HeLa cells were used as positive controls and were treated similarly. A necropsy performed three weeks post-injection revealed no local or distant lesions in the 10 rats treated with EBx cells while all animals injected with the HeLa cells showed tumoral lesions (evolutive nodules) at the injection sites as well as metastases (Fig. 12).

Together with the *in vitro* genetic stability of the EBx cells, this *in vivo* observation confirm that VIVALIS EBx cells display unique properties that make them a safe, regulatory-compliant cell substrate candidate for the production of viral vaccines.

Figure 12: EBx cells are non-tumorigenic in newborn immuno-suppressed rats



	HeLa	EB14
Lethality	1 death	No death
Macroscopic examination	9/9 evolutive nodules (mean diameter: 2cm; mean thickness: 1.1cm)	No nodules
Necropsy examination	large nodules at injection sites + pulmonary nodules in 9/9 rats	No pathological signs

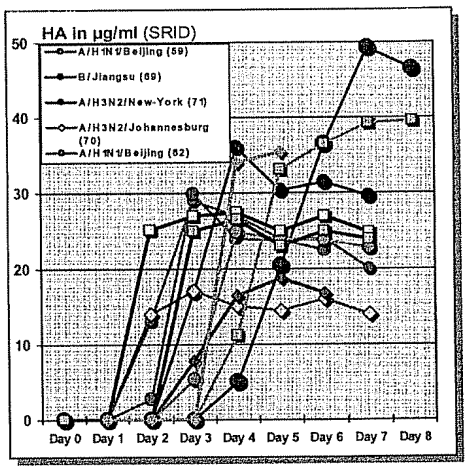
EBx® CELL FOR THE PRODUCTION OF SUBSTANCES OF INTEREST

4.1 – Production of viral vaccines

The susceptibility of EBx® cells to infection and propagation of a series of viral vaccine candidates was also investigated by the inventors. Chicken and duck EBx® cells are highly susceptible to a large series of viruses, such as orthomyxovirus (i.e influenza virus) (Fig. 13), poxvirus (i.e modified vaccinia Ankara) (Fig.14), paramyxovirus (i.e Newcastle Disease virus) (Fig. 15), reovirus (Fig. 15).

Figure 13 :

13A) - Replication of different influenza virus strains A & B in chicken EBx cells cultured in suspension in serum free, exogenous growth factors free, cell culture medium in 3L stirred tank bioreactor.



13B) - Replication of different influenza virus strains A & B in duck EBx cells cultured in suspension in serum free, exogenous growth factors free, cell culture medium in 3L stirred tank bioreactor

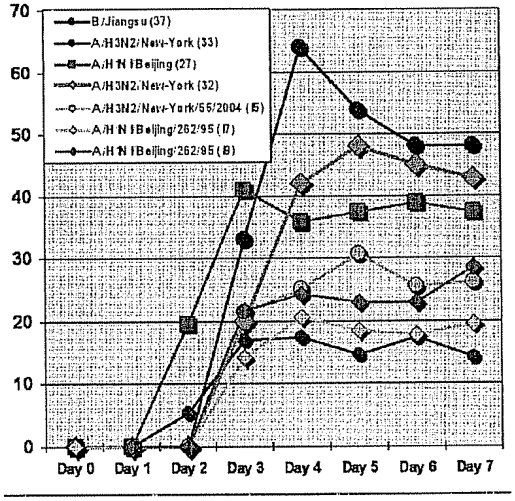
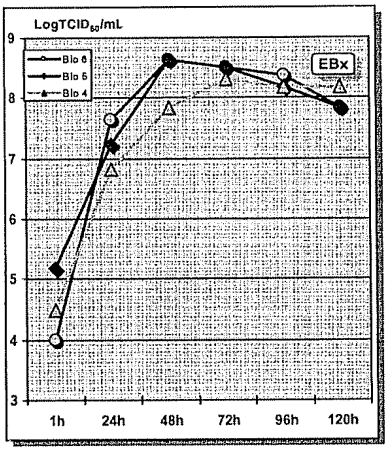


Figure 14 :

14A) - Replication of Modified Vaccinia Ankara (MVA) in chicken EBx cells cultured in suspension in serum free, exogenous growth factors free, cell culture medium in 20L stirred tank bioreactor.



14B) - Replication of MVA in duck EBx cells cultured in suspension in serum free, exogenous growth factors free, cell culture medium in 3L stirred tank bioreactor

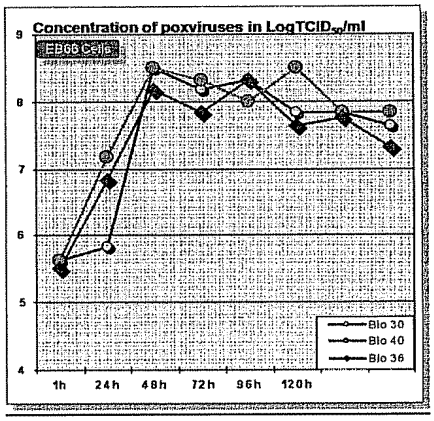
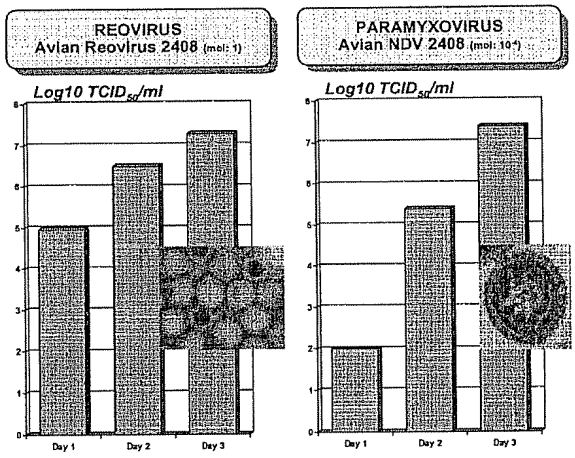


Figure 15 :
Replication of reovirus and Newcastle Disease Virus (NDV) in chicken EBx cells cultured in suspension in serum free, exogenous growth factors free, cell culture medium in tissue culture flask.



The results reported in Table 2 summarize the viruses for which a replication was already demonstrated in chicken or duck EBx cells.

Table 2: List of viruses that do replicate in EBx® cells
Dark green: Viruses for which the replication was demonstrated in chicken EBx and duck EBx cell lines. Light green: Viruses for which the replication was demonstrated only in chicken EB14 cells so far. White: Viruses for which the replication were not yet evaluated by the inventors.

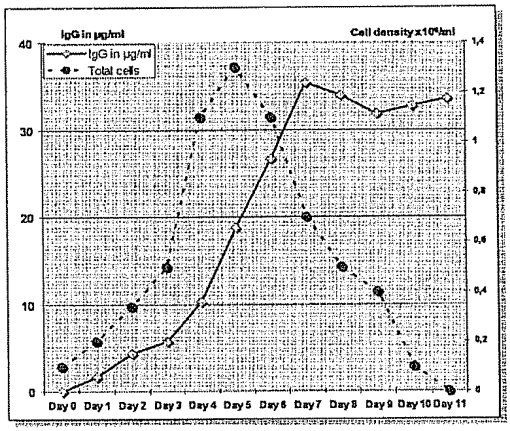
HUMAN HEALTH	ANIMAL HEALTH		
	AVIAN	SWINE, EQUINE, BOVINE	CAT / DOG
Influenza	Influenza	Influenza virus (swine, equine)	Canine Distemper
Avian Newcastle Disease (oncolytic vaccine)	Avian Reovirus	Eastern Equine Encephalitis	Canine Parainfluenza
Measles	Fowlpox	Western Equine Encephalitis	
Mumps	Wt and rec. Poxviruses (Canary, chicken, Pigeon ...)	Equine encephalomyelotis	
Rubella	Egg Drop Syndrome	Bovine Parainfluenza	
Rabies	Newcastle Disease (La Sota strain & mesogenic strain...)	Bovine Ibaraki	
Smallpox	Infectious Bursal Disease	Rabies	
ALVAC (canary pox)	Avian adenovirus (type I, II & III)	Swine japanese encephalitis	
MVA	Polyoma (type I & II)		
Fowlpox	Herpes (Pigeon, Turkey, falcon, psittacine...)		
Thick-Borne encephalitis	Infectious Bronchitis		
Yellow fever	Encephalomyelotis		
CELO (Avian adenovirus)	Chicken anemia		
Sindbis	Marek's disease		
Semliki Forest	Duck Parvovirus		
Venezuelan EEV			

4.2 – Production of recombinant proteins

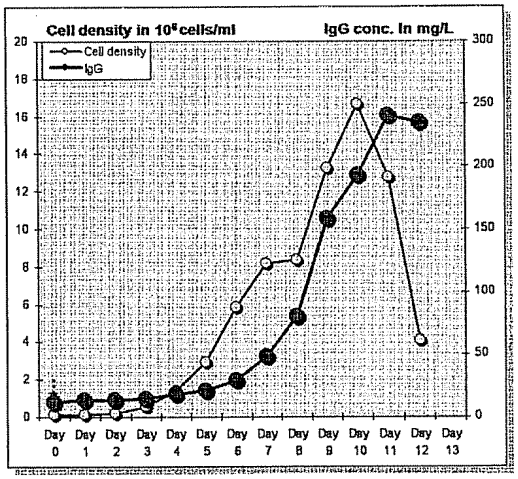
EBx® cells are amenable to easy genetic modification to express recombinant proteins. The inventors demonstrate the efficient and stable production of therapeutic proteins (i.e monoclonal antibody) in serum free medium in stirred-tank bioreactors (Fig. 16).

Figure 16 :

16A) – Production of monoclonal antibody in chicken EBx cells cultured in suspension in serum free, exogenous growth factors free, cell culture medium in 3L stirred tank bioreactor.



16B) – Production of monoclonal antibody in duck EBx cells cultured in suspension in serum free, exogenous growth factors free, cell culture medium in 3L stirred tank bioreactor.



APPENDIX 1

EXAMPLE 1: chicken EBv13 cell line from SPF chicken strain VALO

1. 1.1 - RAW MATERIAL

Eggs

Specific Pathogen Free (SPF) strain called Valo. The valo strain is a white Leghorn strain produced and delivered by Lohmann from Germany. Those SPF chicken eggs, supplied with a certificate of analysis, are tested for: CAV, Avian adenoviruses (group 1, serotypes 1-12 and group 3), EDS, Avian Encephalomyelitis Virus, Avian Leukosis Viruses/RSV (including Serotype ALV-J), Avian Nephritis Virus, Avian Reoviruses, Fowlpox Virus, Infectious Bronchitis Virus, Infectious Bursitis Virus (IBDV), Infectious Laryngo Tracheitis Virus, Influenzavirus Typ A, Marek's Disease Virus, Mycoplasmosis (Mg + Ms), Mycobacterium avium, Newcastle Disease Virus, Reticuloendotheliosis Virus, Salmonella pullorum, Other Salmonella Infections, Avian Rhinotracheitis Virus (ART), Hemophilus paragallinarum. Valo chicken eggs were only submitted to a disinfection with the decontaminant to avoid any risk of contamination linked to the manipulation of eggs during the transport.

Feeder cells

In the first step of the process of establishment of EBv13, cells from murine origin (STO cells) were used as feeder layer to maintain the pluripotency of chicken stem cells. Those feeder cells are mitotically inactivated by gamma irradiation (45 to 55 Grays) before seeding on plastic. This dose of irradiation is a sub-lethal dose that induces a definitive arrest of the cell cycle but still permits the production of growth factors and extracellular matrix, necessary for the promotion of the cell growth of non differentiated cells.

The STO cell line was derived by A. Bernstein, Ontario Cancer Institute, Toronto, Canada from a continuous line of SIM (Sandos Inbred Mice) mouse embryonic fibroblasts and it was supplied by the American Type Culture Collection (ATCC) (STO Product number: CRL-1503, Batch number 1198713). Fresh feeder layers

were prepared twice a week, in general on monday and thursday. Exponentially cells were dissociated and counted. A part of cells were seeded for maintenance of viable cultures and another part was irradiated. For irradiation, we prepared a cell suspension at $10x10^6$ cells/mL in tubes. Cells were exposed to a 45 to 55 grey dose and were seeded on plastic. After seeding, dishes or plates coated with inactivated feeder cells were used during a maximum of 5 days.

Medium

DMEM- HamF12 (Cambrex, Cat n° BE04-687)

Optipro medium (Invitrogen, Cat n° 12309)

EX-CELL ^{TM} 65195, 60947 and 65319 (SAFC, customized medium)

Additives

Glutamine (Cambrex, Cat n° BE17-605E)

Pencillin/streptomycin (Cambrex, Cat n° BE17-602E))

Non essential Amino Acids (Cambrex, Cat n° BE13-114E)

Sodium pyruvate (Cambrex, Cat n°BE13-115)

Vitamines (Cambrex, Cat n° 13-607C)

Beta Mercapto Ethanol (Sigma, Cat n° M7522)

Buffer and fixators

PBS 1X (Cambrex, Cat n° BE17-516F)

Paraformaldehyde 4% (Sigma, Cat n° P6148)

KCl 5,6% (Sigma, Cat n° P9333)

Methanol/ Acetic acid (3/1) : Methanol (Merck, Cat n° K34497209 ; Acetic acid Sigma Cat n°A6283)

Colcemid, Karyomax (Gibco, Cat n° 15212-046)

Cryoprotective agent

Dimethyl Sulfoxyde (DMSO) (Sigma, Cat n° D2650)

Factors

Two different recombinant factors were used:

- ❒ Recombinant Human Ciliary Neurotrophic Factor (CNTF) (Peprotech Inc, Cat n° 450-13)
- ❒ Recombinant Human Insulin Like Factor I (IGF1) (Peprotech Inc, Cat n° 100-11)

The two factors were produced in E. Coli bacteria.

Fetal Bovine Serum

Non irradiated Fetal Bovin Serum (FBS) (JRH, Cat n° 12103)

The non irradiated serum used in the program was collected and produced in United States. Animals used for collection were USDA inspected and acceptable for slaughter. It was added in the medium during avian stem cells culture. This batch was not submitted to irradiation to avoid the destruction of critical proteins or components identified as essential for the maintenance of stem cells in culture.

Irradiated serum (JRH, Cat n° 12107)

The irradiated batch used in this program was also collected in United States. This irradiated batch was added as supplement in the DMEM medium used for the culture of STO or FED cells (feeder cells). Those cells do not require as stem cells a specific quality of serum for growth and maintenance in culture. To minimize high concentration of serum in the medium we have adapted the STO cells to grow in presence of 4% of FBS only.

Dissociating agents

- ***Pronase*** (Roche, Cat n° 165 921)

Pronase is a recombinant protease manufactured by Roche Diagnostics, Germany, used for the dissociation of adherent avian stem cells.

- ***Trypsine EDTA (Cambrex, cat n° BE17-161E)***

Trypsine is used for the dissociation of STO or FED cells and at late passages for the dissociation of avian cells adapted to Serum Free Medium. This enzyme of porcine origin is manufactured aseptically according to cGMP referential conditions by a validated sterile filtration method and tested according to current E.P. The raw

material, irradiated prior to formulation, is tested for porcine parvovirus in strict compliance with 9/CFR 113.53.

- ***Non enzymatic cell dissociation solution (Sigma, Cat n° C5914)***

This agent of dissociation is a ready to use formulation used to gently detach cells from the growing surface of the culture vessel. The formula contains no protein, and allows dislodging of cells without use of enzymes. Cellular proteins are preserved making possible immunochemical studies that are dependent upon the recognition of cell surface proteins. This enzyme was used to detach cell before FACS analysis of biological markers like EMA-1 (Epithelial Membrane Antigen 1) and SSEA1 (Stage Specific Embryonic antigen-1).

2. 1.2 - PROCESS OF ESTABLISHMENT OF CHICKEN EBv13 CELL LINE

Eggs are opened, the yolk were separated from the albumen during the opening. The embryos were removed from the yolk either directly with the aid of a Pasteur pipette, or with the aid of a small absorbent filter paper (Whatmann 3M paper), cut out beforehand in the form of a perforated ring with the aid of a punch. The diameter of the perforation were about 5 mm. These small rings were sterilized using dry heat for about 30 minutes in an oven. This small paper ring is deposited on the surface of the yolk and centered on the embryo which is thus surrounded by the paper ring. The latter is then cut out with the aid of small pairs of scissors and the whole removed is placed in a Petri dish, filled with PBS or with a physiological saline. The embryo thus carried away by the ring were cleaned of the excess yolk in the medium and the embryonic disk, thus free of the excess vitellin, is collected with a Pasteur pipette.

The chicken Valo embryos were placed in a tube containing physiological medium (1X PBS, Tris Glucose, medium, and the like). The Valo embryos were then mechanically dissociated and inoculated on a layer of feeder STO cells into complete culture medium at 39°C. The feeder cells were seeded in flask at around $2.7x10^4$ cell/cm^2. The complete culture medium is composed of basal commercial medium DMEM-Ham F12 supplemented with 10% fetal calf serum, with IGF1 and CNTF at a

AA

final concentration of 1ng/ml, and with 1% non-essential amino acids, with 1% of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 1 mM, with beta-mercapto-ethanol at a final concentration of 0.2 mM, glutamine at a final concentration of 2.9 mM, with an initial mixture of antibiotics containing penicillin at a final concentration of 100 U/ml and streptomycin at a final concentration of 100 μg/ml. Rapidly after the first passages of the cells, the mixture of antibiotics is no longer added to the medium. The expression rapidly is understood to mean after the first 3 to 5 passages in general.

When the avian ES cells from chicken Valo embryos is passaged from a culture flask to another, the seeding of culture flasks was performed with around between 7 x $10^4/cm^2$ to 8 x $10^4/cm^2$ of avian ES cells in the complete culture medium. Preferably, the seeding is made with around 7.3 x $10^4/cm^2$ (4 x 10^6 cells/55cm^2 or 4 x 10^6 cells/100 mm dish). The avian cells, preferably the avian embryonic cells of step a) are cultured during several passages in the complete medium. At passage 15, the complete medium was depleted in growth factors IGF1 and CNTF. The depletion is made directly in one step, from one passage to another. The embryonic stem cells, preferably the avian embryonic cells are cultured during several passages in the complete medium without IGF1 and CNTF growth factors.

Then depletion of feeder cells were performed after the depletion of growth factors IGF1 and CNTF by a progressive decreasing of feeder cells concentration over several passages. Practically, the same concentration of the feeder cells were used for 2 to 4 passages, then a lower concentration of the feeder cells were used for an additional 2 to 4 passages, and so on. The flask were originally seeded with around 2.7 x10^4 feeder cells/cm^2, then around 2.2 x 10^4 feeder cells/cm^2, then around 1.8 x 10^4 feeder cells/cm^2, then around 1.4 x 10^4 feeder cells/cm^2, then around 1.1 x 10^4 feeder cells/cm^2, then around 0.9 x 10^4 feeder cells/cm^2, then around 0.5 x 10^4 feeder cells/cm^2 . Then the flask were seeded with 6.5 x 10^4 avian cells/cm^2 to 7.5 x 10^4 avian cells/cm^2 and without feeder cells. The depletion of feeder cells started at around passage 21 and ended at around passage 65. During the depletion of feeder cells, the chicken Valo ES cells were seeded in culture flask at a lower concentration

than in step a), about around 4 x 10^4 cell/cm^2 to 5 x 10^4 cell/cm^2. In the hypothesis that Valo ES cells were not in good shape following a decrease of feeder cells concentration in the flask, then the avian cells are cultured for additional passages with the same feeder cells concentration before to pursue the feeder cells depletion.

The serum depletion were performed after the growth factor and the feeder cells depletion. At the beginning of serum depletion, the culture medium were composed of basal commercial medium DMEM-HamF12 supplemented with 10% fetal calf serum and with 1% non-essential amino acids, with 1% of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 1 mM, with beta-mercaptoethanol at a final concentration of 0.2 mM, glutamine at a final concentration of 2.9 mM. The chicken Valo cells were adapted to the growth in a serum free medium culture in a two steps process: first, the chicken Valo cells were rapidly adapted to a culture medium composed of commercial serum free medium (SFM), preferably ExCell 60947 (SAFC Biosciences) supplemented with 10% fetal calf serum and with 1% non-essential amino acids, with 1% of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 1 mM, with beta-mercaptoethanol at a final concentration of 0.2 mM, glutamine at a final concentration of 2.9 mM. Once this rapid adaptation to a new medium (DMEM-HamF12 to Excell 60947) was performed, a second step is performed consisting of a slow adaptation to decreasing concentration of animal serum in the SFM medium were initiated. Serum depletion was performed by a progressive decreasing starting from 10% serum, then 7.5%, then 5%, then 2.5%, then 1.25%, then 0,75% of serum concentration in SFM cell culture medium to finally reach 0% serum in SFM cell culture medium. Serum depletion started at passage 103 and ended at passage 135.

At the end of the process of deprivation of serum when the remaining concentration of serum in SFM medium was either 0.75% or 0%, the adaptation of anchorage-dependent EBv13 cells to suspension culture started. Among the several attempts performed to isolate anchorage-independent EBv13 isolates, 62.5% of the attempts were successful and allow to get different isolates of suspension EBv13 cells. One isolate of EBv13 cells were selected according to the population doubling time

(around 18h), the optimal cell concentration into flask culture (around 4 million cell/ml), the cell viability, the cell culture homogeneity (presence and size of cells clumps) and the easiness to manipulate the cells (Figure 1).

At the end of serum depletion, anchorage dependent chicken Valo cells, named EBv13 were able to grow in absence of grow factors, in absence of feeder cells, in serum free medium. EBv13 Cells were then adapted to growth at 37°C, by progressively decreasing cell culture temperature of 0.5°C /day.

EXAMPLE 2: Duck EBx cell line EB26

3. 3.1 - RAW MATERIAL

Duck Eggs, Feeder cells, additives, Buffers and Fixators, Cryopreservative agents, Fetal Calf Serum & dissociating agents (Idem as Example 2).

Duck eggs from Peking strains GL30 were used.

Medium

Medium EX-CELL 65319, 63066 and 66444 (SAFC, customized medium)
Medium GTM-3 (Sigma, Cat n° G9916)
DMEM (Cambrex, Cat n° BE 12-614F)

Factors

Six different recombinant factors were used:

- ❒ Recombinant Human Ciliary Neurotrophic Factor (CNTF) (Peprotech Inc, Cat n° 450-13)
- ❒ Recombinant Human Insulin Like Factor I (IGF1) (Peprotech Inc, Cat n° 100-11)
- ❒ Recombinant Human Interleukin 6 (IL6) (Peprotech Inc, Cat n° 200-06)
- ❒ Recombinant Human soluble Interleukin 6 receptor (sIL6r) (Peprotech Inc, Cat n° 200-06 R)
- ❒ Recombinant Human Stem Cell Factor (SCF) (Peprotech Inc, Cat n° 300-07)

❒ Recombinant Human basic Fibroblast Growth Factor (bFGF) (Peprotech Inc, Cat n° 100-18B)

All those factors, except IL6r, are produced in *E. Coli* bacteria. Soluble IL6r is expressed in transfected HEK293 cells.

3.2 - PROCESS OF ESTABLISHMENT OF DUCK EBx CELL LINE EB26

The duck embryos were collected as previously described with EB66. The duck embryos were placed in 50 mL tubes containing PBS 1X. The duck embryos were then mechanically dissociated, washed in PBS, and seeded on an inactivated layer of feeder STO cells into complete culture medium at 39°C, 7,5% CO_2. The feeder cells were seeded in 6 well plates or dishes at around 2,7 $x10^4$ cell/cm^2. The complete culture medium is composed of serum free medium GTM-3 supplemented with 5% fetal bovine serum, with IGF1, CNTF, Il-6, Il-6R, SCF and FGF at a final concentration of 1ng/ml, and with 1% non-essential amino acids, with 1% of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 0,1 mM, with beta-mercapto-ethanol at a final concentration of 0.5 mM, glutamine at a final concentration of 2,1 mM, penicillin at a final concentration of 100 U/ml, streptomycin at a final concentration of 100 µg/ml and yeastolate 1X. Rapidly after the first passages of the cells, the mixture of antibiotics is no longer added to the medium. The expression rapidly is understood to mean after the first 3 to 9 passages in general. The duck ES cells were cultured in the complete medium up to passage 9. After passage 9, the complete medium is partially depleted in factors. Thus, between passage 10 and 13, SCF, IL6, IL6r and bFGF were removed for the medium and only recombinant IGF1 and CNTF were maintained at a concentration of 1 ng/mL. A simultaneous decease of concentration of IGF1 and CNTF is secondly performed between passage 13 and 16 to finally obtain cells able to grow without recombinant factors at passage 17. The factor depletion were made by a progressive adaptation to lower concentrations of factors. When the duck ES cells from Pekin duck embryos were passaged from a culture dish to another, the seeding of culture dish was performed with around between 7 x 10^4/cm^2 to 12 x 10^4/cm^2 of duck ES cells in the complete culture medium. Preferably, the seeding is made with around

7.3 x 10^4/cm^2 (4 x 10^6 cells/55cm^2 or 4 x 10^6 cells/100 mm dish). After depletion of recombinant factors, a decrease of yeastolate were performed at passage 23 reaching the final concentration at 0,5X. Then, after passage 31, depletion of feeder cells were performed by a progressive decrease of feeder cells concentration over several passages.. The dishes were originally seeded with around 2,7 x10^4 feeder cells/cm^2, then around 1,8 x 10^4 feeder cells/cm^2 between passage 32 and 38, then around 1,4 x10^4 cells/cm^2 between passage 39 and 44, then around 1 x 10^4 feeder cells/cm^2 between passage 45 and 47, then around 0,7 x 10^4 feeder cells/cm^2 between passage 48 and 50, and finally from passage 51 dishes were seeded only with avian cells and without feeder cells,. At the end of the feeder depletion, the dishes are seeded with 9 x 10^4 avian cells/cm^2 to 12,7 x 10^4 avian cells/cm^2. The depletion of feeder cells started at passage 32 and ended at passage 51. During the depletion of feeder cells, the duck ES cells are seeded in culture dishes at a higher concentration than in step a), about around 9 x 10^4 cell/cm^2 to 12,7 x 10^4 cell/cm^2. After several passages without feeder cells, growth parameters (Population Doubling Time (PDT) and Density) were studied to confirm cell stability and robustness and to initiate the cell growth as suspension. Cells are considered as enough robust to be submitted to a culture in suspension if, PDT is lower than around 40 hours and cell density higher than around 26x10^4 cells/cm^2. Moreover, cells morphology should be: round, refringent, very small and the cells shall not attached to the plastic dish too much.

In the case of the EB26 cell development, culture in suspension were initiated at passage 53. 7 x10^6 cells were transferred in a Ultra Low attachment dish and maintained under constant agitation at around 50 to 70 rpm. For the next passages, cells were seeded in T175 flasks (Sarsted, ref 831812502) at a concentration comprise between 0,4 to 0,5x10^6 cells/mL. Following a short period of adaptation to the new conditions of culture, cells PDT decreased from around 160 H to 40 hours. Regarding this good evolution, at passage 59, a new set of deprivation was performed. Thus vitamins, sodium pyruvate, beta-mercaptoethanol and non essential amino acids were removed. Thus after passage 59, the culture medium was supplemented with 5% FBS, 0,5 X yeastolate and 2,5 mM glutamine only. The

serum depletion is performed on cell suspensions already depleted in growth factor, feeder cells, vitamins, non essential amino acids, sodium pyruvate and beta-mercaptoethanol. Serum depletion was performed by a progressive decreasing starting from 5% serum, then 2.5%, then 1,5%,of serum concentration in SFM cell culture medium to finally reach 0% serum in SFM cell culture medium. Serum depletion started at passage 61 and ended at passage 79. At the end of serum depletion, anchorage independent duck EB26 cells were able to grow at 39°C in absence of grow factors, in absence of feeder cells, in serum free medium. EB26 cells were then adapted to growth in absence of 0.5X yeastolate at 37°C, by decreasing cell culture temperature at passage 80.

After the obtaining of EB26 cells that are able to grow at 37°C in the SFM GTM-3 supplemented by 2,5 mM glutamine, some further adaptation were made by dilution or progressive adaptation on new SFM formulations as Excell 63066, Excell 66444, Excell CHO ACF. The subcloning of suspension duck EB26 cell could also realized in presence or absence of yeastolate.

EXAMPLE 3: EBx Cell Lines Characterization

4. 5.1 - CHICKEN VALO EBv13 CELLS CHARACTERIZATION

5.1.1 - Telomerase activity

Telomerase detection is achieved by using the *Telo* TAGGG Telomerase PCR ELISA developed by Roche Applied Science (Telomeric Repeat Amplification Protocol (TRAP) – Cat. No. 11 854 666 910) according to the supplier protocol. The *Telo* TAGGG Telomerase PCR ELISA allows amplification of Telomerase-mediated elongation products combined with non radioactive detection following an ELISA protocol. The assay is valid if absorbance value of the negative control is less than or equal to 0.25 $A_{450nm} - A_{690nm}$ and if absorbance value of the positive control is higher than or equal to 1.5 $A_{450nm} - A_{690nm}$ when using 1×10^3 cell equivalents in the assay. Samples are regarded as telomerase positive if the difference in absorbance is higher than 0,2 $A_{450nm} - A_{690nm}$ units. Two controls were used: the negative control is

murine fibroblasts (FED cells) and the positive controls are FGB8 cells (Embryonic Stem cells established by Vivalis from 129 SV mouse embryos) and chicken EB14-O74 cells previously established in WO03/076601. .
Results obtained are summarized on the figure N°2 . EBv13 cells do express high level of telomerase. At passage p193 and 195, the telomerase activity is equivalent to the one of chicken EB14-O74 cells.

5.1.2 - ES cells biological markers

Embryonic stem cells are characterized by the expression of biological markers expressed on the cell membrane. The expression of EMA-1 (Epithelial Membrane Antigen-1) and SSEA-1 (Stage Specific Embryonic Antigen-1) on EBv13 cells were evaluated by FACS analysis. After 10 minutes of fixation with PFA 4% (Para-formaldehyde), cell samples and controls are rinsed and pre-incubated with monoclonal antibodies specific of EMA-1 or SSEA-1. A second antibody conjugated to FITC is used for detection of cells expressing the 2 biological markers selected. Samples were analyzed by flow cytometry using a FACS (Flow Activated Cell Sorter) from Coulter.
FACS analysis was done on mouse fibroblasts cells (FED cells) as a negative control, murine ES FGB8 cells as a positive control, chciken EB14-O74 cells as a positive control EBx cells and EBv13 cells. As expected FED cells do not express biological markers whereas FGB8 and EB14-O74 cells present an important staining, respectively of, 60,13% and 78,7 for EMA-1 and 94,45 % and 95% for SSEA-1 (data not shown). Chicken valo EBv13 cells population do not present any staining for EMA1 (2%) and a very light one for SSEA-1(22%).

5.1.3 - Karyotype

Karyotype analysis was performed to check the cell diploidy and the avian origin of EBv13 cells. Cells in the exponential phase of growth were harvested and treated 2 hours by colcemid (0,02µg/mL). After washing and centrifugation, an hypotonic choc is performed on cells with KCl (0,56%) during 20 minutes. Subsequently, EBv13 cells were fixed in methanol/acetic acid (3/1) and stored overnight at -20°C. The day after,

metaphasis were spotted on glass, stained by a wright/giemsa solution and observed under microscope. Several series of metaphases were observed confirming the chicken origin of EBv13 cells. No evidence of polyploidy is observed;

8. I further declare that all statements made herein of my own knowledge are true and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 30th 2008 By: Mehtali